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STACKING OF SAFRANINE IN LIPOSOMES DURING VALINOMYCIN-INDUCED EFFLUX OF POTASSIUM IONS

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SUMMARY

Liposomes were prepared from phosphatidylcholine and cardiolipin in a KCl medium and suspended in a choline chloride medium with safranine. When efflux of K^+ was induced by valinomycin, spectral shifts characteristic of stacking were observed. Ca^{2+} inhibited the rate of stacking in a competitive manner with a K_i of about 200 μM, while La^{3+} was about 10 times more potent. When liposomes were prepared from phospholipids with a higher ratio of cardiolipin to phosphatidylcholine the inhibition was more potent. No effect on the stacking phenomena was seen when Ca^{2+} was added after the stacking was completed. When Ca^{2+} or an organic cation with four charges, spermine, was trapped in the intraliposomal compartment, no significant change in the rate of stacking was seen. However, the extent of stacking was decreased. It is suggested that safranine is driven by a diffusion potential to a site that is inaccessible to Ca^{2+} in the medium, presumably to the inner boundaries of the liposomal membranes.

INTRODUCTION

Safranine, a cationic dye, is bound in an energy-dependent way by mitochondria and submitochondrial particles to internal binding sites [1, 2]. The spectral shifts recorded in this process have been interpreted as being due to stacking of the dye [1, 2]. This transport is inhibited competitively by Ca^{2+} and has thus been suggested to occur by the same transport system that transports Ca^{2+} into mitochondria [1, 2]. Similar phenomena have been reported for safranine bound by bacterial vesicles [3]. We now demonstrate that safranine is taken up by liposomes and exhibits stacking when a diffusion potential is induced.

METHODS AND MATERIALS

Liposomes were prepared essentially as described by Papahadjopoulos and co-workers [4], in 130 mM KCl, 20 mM Tris–Cl (pH 7.5) except that 50 mg/l of

Abbreviation: EGTA, ethyleneglycol bis (α-aminoethylether)-*N*,*N*′-tetraacetic acid.

butylated hydroxyanisole was included to prevent auto-oxidation. The spectral shift of safranine was measured with an Aminco DW2 spectrophotometer using the wavelength pair 524–554 nm.

For preparation of liposomes Soya phosphatidylcholine (NB Co., Cleveland, Ohio 44128) and 10 % (w/w) cardiolipin (Sylvana Company, Millburn, New Jersey 07041) were used. Safranine was obtained from Merck AG, Darmstadt, GFR, and valinomycin from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Spectral shifts of safranine

When valinomycin is added to liposomes containing KCl and suspended in choline chloride the spectrum of safranine is changed in a way typical of stacking (Fig. 1). If the liposome concentration is increased the extent of the decrease in absorbance is less (not shown), probably because of destacking [2]. No such spectral

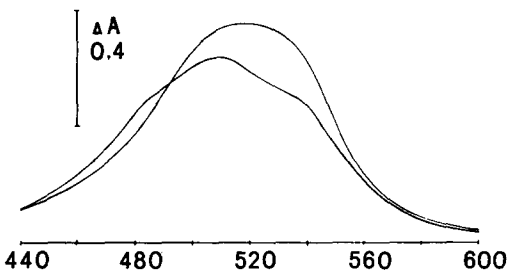

Fig. 1. Spectral shift of safranine after addition of valinomycin in the presence of liposomes. Liposomes were suspended to a concentration of 0.2 mM phosphate in a medium containing 60 mM choline chloride, 140 mM sucrose, 20 mM Tris · Cl (pH 7.5) and 40 μM safranine. The spectrum was drawn in the absence (upper trace) and presence (lower trace) of 40 ng/ml valinomycin.

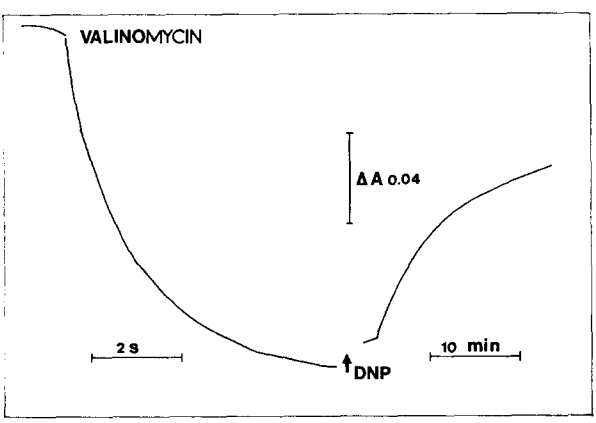

Fig. 2. Kinetics of safranine stacking induced by a K^+ diffusion potential created by valinomycin. Conditions as in Fig. 1. Additions: 40 ng/ml valinomycin and 0.8 mM 2,4-dinitrophenol (DNP).

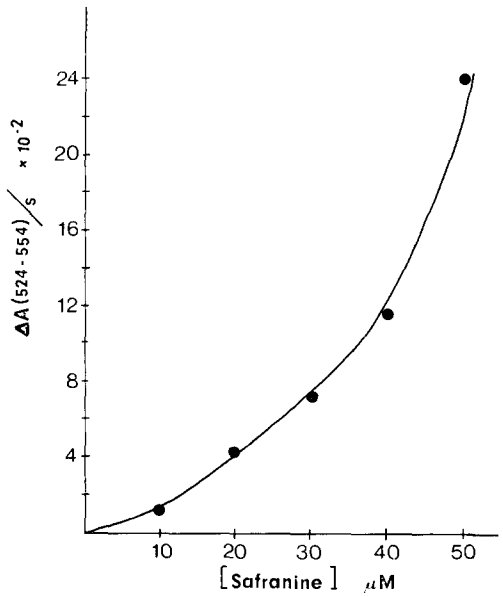

Fig. 3. Kinetics of safranine stacking as a function of the safranine concentration. The initial rate of safranine uptake was plotted from similar plots to those in Fig. 2, at different safranine concentrations. Other conditions the same as in Fig. 1.

changes were seen when, in control experiments, the liposomes were suspended in KCl medium. It is thus evident that the stacking is associated with the creation of a membrane diffusion potential due to the valinomycin-induced efflux of K^+. Since safranine is a cation with one charge shielded by hydrophobic groups, it should readily penetrate the hydrophobic core of the membrane. Indeed we found a high partition ratio (approx. 100) between heptane (or chloroform) and the medium used. It seems highly probable that the shift is produced as the dye accumulates at

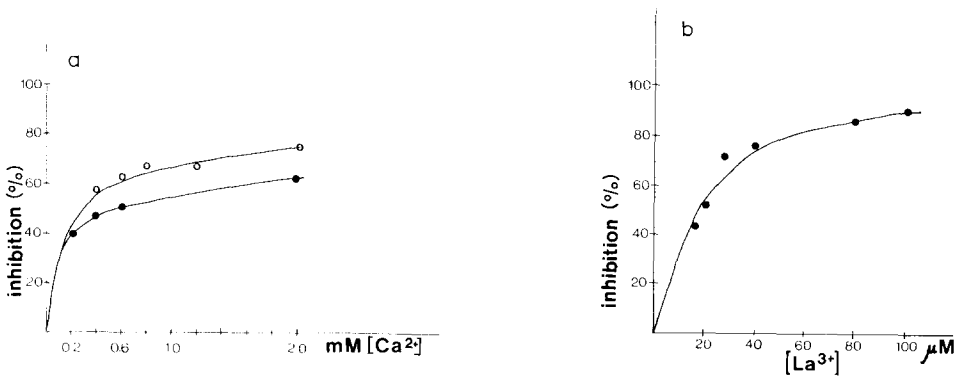

Fig. 4. Inhibition of safranine stacking by Ca^{2+} and La^{3+}. Percent inhibition of the initial rate of safranine or stacking is plotted against the Ca^{2+} or La^{3+} concentration. (a) Ca^{2+} inhibition: (○) 20 μM safranine; (●) 40 μM safranine. (b) La^{3+} inhibition: safranine 40 μM. Other conditions as in Fig. 1.

the inner boundary of liposome membranes as a response to the membrane potential [5].

Kinetics of safranine transport

Fig. 2 shows the kinetics of the stacking. Valinomycin induced a change with a $T_{\frac{1}{2}}$ of about 1 s. Addition of a proton-conducting agent like 2,4-dinitrophenol caused a slow reversal of the response. These changes correspond to those occurring in mitochondria upon "energization" and "de-energization" [1]. In Fig. 3 the initial rate of the valinomycin-induced shift is plotted against the concentration of safranine. A highly sigmoidal curve is obtained, with no indication of saturation at the safranine/liposome ratios used. The sigmoidicity is probably due to nonlinear correlation of stacking to the concentration of the dye at the appropriate sites (compare refs. 1 and 2).

Inhibition of safranine transport by Ca^{2+} and La^{3+}

The stacking of safranine is inhibited by Ca^{2+} and the trivalent rare earth La^{3+} (Fig. 4). The inhibition by Ca^{2+} appears to be competitive, because Ca^{2+} inhibits more extensively at lower safranine concentrations (Fig. 4a). Ca^{2+} is bound to acidic phospholipids [6, 7] but is not able to penetrate liposomes in the absence of a divalent cation ionophore (unpublished results). It is therefore most probable that the inhibition by these cations is due to interference with the penetration of safranine through the outer boundary of the membranes. The stacking probably occurs at the inner surface of the membranes because Ca^{2+} is without effect when added after the spectral shift has occurred (not shown). Fig. 4a also shows that the inhibition plots for Ca^{2+} are strongly biphasic, and this is probably due to the fact that two kinds of phospholipids were used for the preparation of liposomes, namely the zwitter-

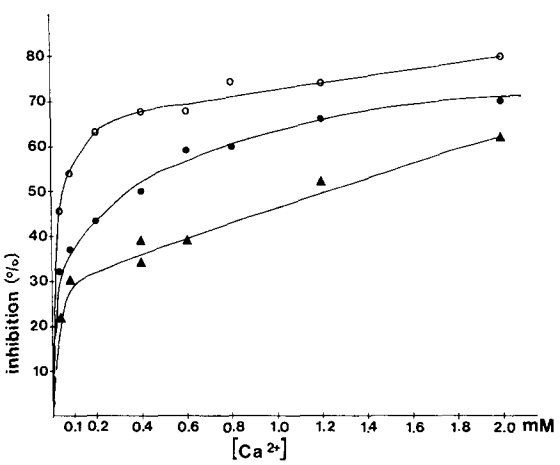

Fig. 5. Inhibition of safranine uptake by Ca^{2+} at different cardiolipin/phosphatidylcholine ratios. (▲) 5 % (w/w) cardiolipin, (●) 10 % cardiolipin, (○) 20 % cardiolipin. Safranine 20 μM. Other conditions as in Fig. 1.

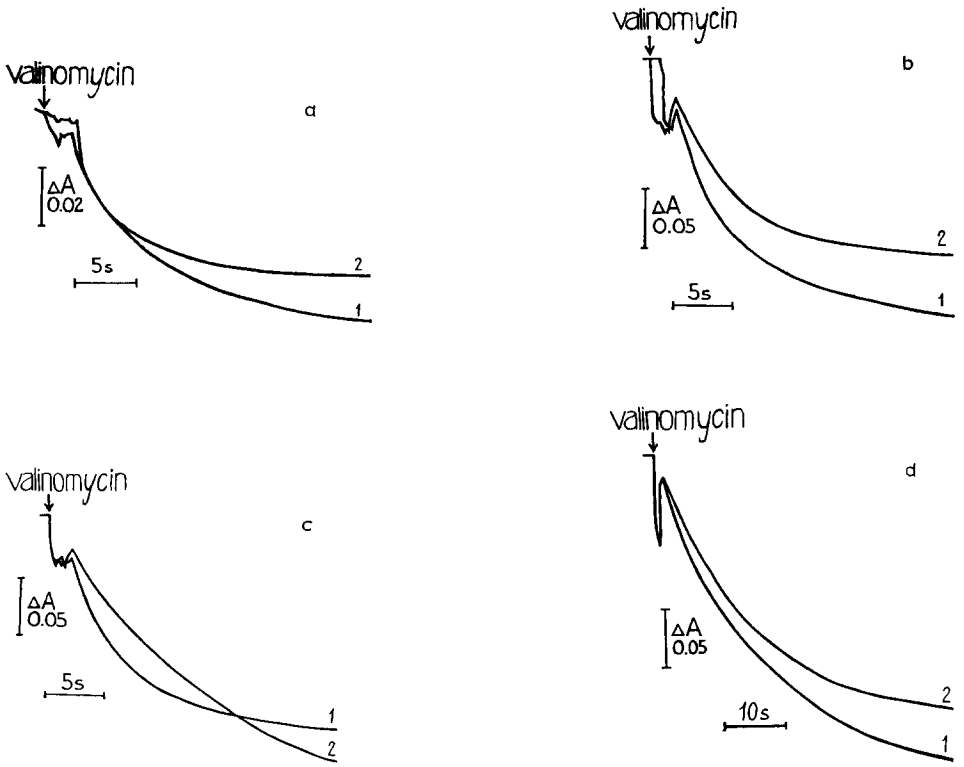

Fig. 6. Decrease in the extent of safranine stacking by internally trapped Ca^{2+} and spermine. Conditions as in Fig. 1. (a) 1, control; 2, liposomes prepared in the presence of 600 μM $CaCl_2$. 1 mM EGTA present in the external medium. Safranine concentration 40 μM. (b) 1, control; 2, liposomes prepared in the presence of 500 μM spermine. (c) 1, control; 2, 40 μM spermine present in the external medium. (d) 1, 40 μM spermine externally; 2, 500 μM spermine internally and 40 μM externally. Safranine concentration 80 μM (b, c, d). Addition: 40 ng/ml valinomycin.

ionic phosphatidylcholine, which has low affinity for Ca^{2+}, and the acidic cardiolipin, with a rather high affinity [8]. Increasing the content of cardiolipin increased the extent of the steep rise in the inhibition plots (Fig. 5).

Inhibition of the extent of safranine stacking by internally trapped Ca^{2+} and spermine

When liposomes are prepared in the presence of 600 μM Ca^{2+} the extent of safranine stacking is decreased (Fig. 6a). This effect is seen both in the presence and in the absence (not shown) of externally added ethyleneglycol bis(α-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), which clearly indicates that the effect observed is due to the internally trapped Ca^{2+}. A similar effect is seen if the organic cation spermine, with four charges, is trapped inside the liposomes (Fig. 6b). Spermine reacts with negative groups of phospholipids. It also decreases the negative surface charge of mitochondria, probably because of its association with the phospholipids [9]. The effect of spermine on the safranine stacking is not due to spermine remaining in the external compartment, since externally added spermine if anything increases the ex-

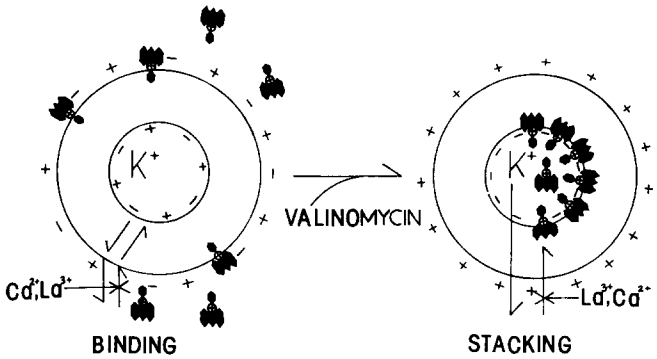

Fig. 7. A schematic picture of the safranine uptake and stacking. When safranine is added to the external medium it is partly bound at the outer surface of the membrane. This binding is inhibited by Ca^{2+} and La^{3+}. When valinomycin is added a membrane potential is generated and the bound safranine is driven to the internal surface of the membrane, where it associates with negative charges of phospholipids. This association is inhibited by internally trapped Ca^{2+} and spermine, owing to competition for the internal binding sites.

tent of safranine stacking (Fig. 6c). Fig. 6d shows the stacking of safranine when spermine is present in both the intra- and extraliposomal compartments.

These experiments indicate that the stacking of safranine occurs at a space inaccessible to externally added Ca^{2+}, presumably the liposomal inner boundary, and that entrance of safranine into the membrane phase is rate limiting (Fig. 7).

It is of interest that the uptake of safranine by liposomes on induction of a membrane potential is so similar to its uptake by "energized" mitochondria. In both cases the experimental findings can be readily explained by a membrane-potential-driven transport of safranine to the inner boundary, where it may associate with negative charges. The spectral changes could also be partly due to accumulation of the dye in the intraliposomal (and intramitochondrial) fluid spaces.

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REFERENCES

1 Dell'Antone, P., Colonna, R. and Azzone, G. F. (1972) Eur. J. Biochem. 24, 553–565
2 Colonna, R., Massari, S. and Azzone, G. F. (1973) Eur. J. Biochem. 34, 577–585
3 Kaback, H. R. (1974) Science 186, 882–892
4 Saha, J., Papahadjopoulos, D. and Wenner, C. E. (1970) Biochim. Biophys. Acta 196, 10–19
5 Liberman, E. A., Topali, V. P. and Zofina, L. M. (1969) FEBS Symposium, Vol. 17, pp. 275–284
6 Carr, C. W. (1971) in Cellular Mechanisms for Calcium Transfer and Homeostasis (Nichols, Jr., G. and Wasserman, R. H., eds.), pp. 41–62, Academic Press, New York
7 Hauser, H. and Dawson, R. M. C. (1967) Eur. J. Biochem. 1, 61–69
8 Shah, D. O. and Schulman, J. H. (1965) J. Lipid Res. 6, 341–349
9 Huunan-Seppälä, A. (1972), M. D. thesis, University of Helsinki, Finland 1–115